SPIN-LABELING STUDIES OF BEEF-LIVER GLUTAMATE DEHYDROGENASE

J-M. JALLON, A. DI FRANCO, F. LETERRIER* and L.PIETTE**

Centre de Génétique Moléculaire du CNRS
91190 Gif-sur-Yvette, France

Received December 20,1976

SUMMARY: Bovine liver glutamate dehydrogenase was spin labeled with a nitroxide derivative of parachloromercuribenzoate. The ESR spectrum was of the immobilized type and the labeling yield 0.6 mole of spin label bound per mole of protomer under standard conditions. The specific activity of the labeled enzyme was not modified but the activation by ADP abolished. Inhibition by GTP was not altered but the ESR spectrum showed that the bound spin label was further immobilized in the presence of GTP and NADPH. In the presence of the coenzyme NADPH, the labeling yield decreased to half its initial value. Such a protection effect was observed neither with NADH nor with ADP.

INTRODUCTION

Two important problems concerning the mechanism of the enzyme beef liver glutamate dehydrogenase (GDH) can be fruitfully approached using the spin labeling technique (1) :

1) the topographical relations between coenzyme and substrate in the active site, following the pioneer work carried out in M. Cohn's laboratory (2).

2) the distance between the active site and the regulatory site for the allosteric inhibitor GTP on one hand, for the activator ADP on the other hand (3).

The prerequisite for such studies is to be able to label an amino acid residue either interacting directly with one of the active ligands or close to their binding sites.

Since many authors have shown that lysine 126 is important for the binding of the 2-oxoglutarate substrate (4-7), we previously labeled this residue using a ketone nitroxide, 2-2'-6-6' tetramethylpiperidone N-oxide (8). The results showed (9) that : 1) this ligand binds to GDH in a very

* F.LETERRIER : Centre de Recherches du Service de Santé des Armées (division de Biophysique), Clamart-France.
**L.PIETTE : Dept. of Biochemistry and Biophysics, Univ. of Hawaii, Honolulu-USA.

immobilized site (through a Schiff base which may be reduced by sodium borohydride); 2) the labeled enzyme has a decreased activity; 3) the labeling is protected by the oxidized substrate 2-oxoglutarate. However conditions could not be found which would allow a labeling higher than 0.12 per protomer making somewhat ambiguous the interpretations. For example, it is not known whether this low level reflects the binding to one site per hexamer, since it is close to one sixth the number of active sites.

In the study presented here, we used another spin label directed towards -SH groups, a derivative of parachloromercuribenzoate (PCMB-SL)(10). Indeed recent crystallographic data have shown that in the coenzyme binding structure of several NAD dependent dehydrogenases there was a cysteine residue in the vicinity of the coenzyme attachements points (11-12). The spin-label experiments seem to show a specific binding to one kind of residue in a rigid environment, the accessibility of which is decreased when the reduced coenzyme is present.

MATERIAL AND METHODS

The beef liver glutamate dehydrogenase was prepared according to Kubo et al. (13). The molar enzyme concentration was measured spectrophotometrically at 279 nm on a Cary 15, using a value of 0.97 $cm^2 .mg^{-1}$ for the absorption coefficient (14) and 56 100 for the molecular weight of one protomer (15). The activity was measured under the following conditions (GDH : 0.5 µM ; NADPH : 150 µM ; 2-oxoglutarate : 15 mM ; NH_4Cl : 0.1 M ; Tris-HCl buffer pH 7.5). The PCMB-SL was synthetized by Dr. C. Hsia in the University of Hawai (10) (cf. Fig. 1). Other ligands were commercial products, nucleotides from Sigma. The electron paramagnetic resonance spectra were recorded with an E_3 Varian spectrometer, using Varian aqueous quartz flat cells, at room temperature.

RESULTS AND DISCUSSION

Labeling was carried out by incubating the enzyme (0.5 - 5 mg/ml) with a small excess of PCMB-SL at room temperature in a 0.1 M phosphate buffer (pH 7.5), for two hours. The reaction time was limited to two hours as the reaction does not seem to proceed any further under these conditions. Then the excess spin label was eliminated either through a G-25 Sephadex column or by an overnight dialysis in the cold room. A small amount of precipitated material was eliminated by centrifugation.

The spectrum shown in Fig. 1 is of a very immobilized type (1). The splitting between the upfield and the downfield peaks is 56 G. With the keton nitroxide, the immobilization was more pronounced (67 G) (9). Each label must thus be rigidly bound to the protein. If one compares this signal with those of the same spin-label at different glycerol concentrations, the tumbling time of the bound label can be evaluated at about 80 nsec.

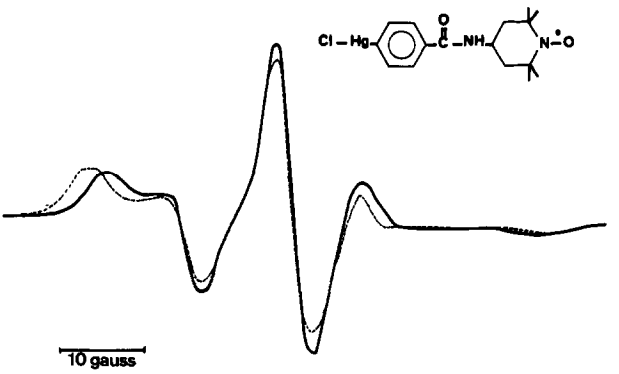

Fig. 1 : ESR spectra of PCMB – nitroxide spin labeled GDH.
Full line, without any ligand ; dotted line, in the presence of 5×10^{-4} M GTP + 5×10^{-4} M NADPH. GDH : 46 µM in 0.1 M phosphate buffer pH 7.5.

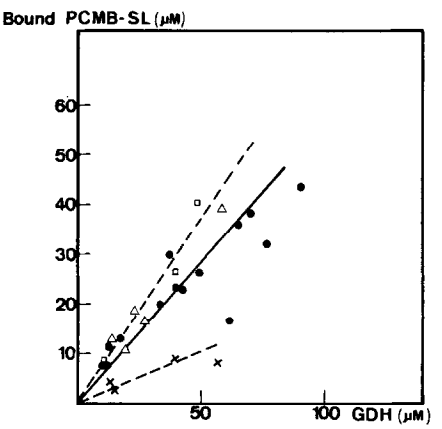

Fig. 2 : Stoichiometry of the PCMB nitroxide spin-labeling.
●, without any added ligand ; x, in the presence of 10^{-4} M NADPH ; ▫, in the presence of 10^{-4} M NADH ; ▲, in the presence of 5×10^{-4} M ADP. All labeling experiments were performed under the experimental conditions described in "Material and Methods", and the reaction time limited to 2 hours.

The spectrum does not present any detectable signal heterogeneity, as was also observed with the ketone nitroxide (9), probably indicating only one type of binding site.

We then quantitated the labeling using the momentum method. Fig. 2 shows that the amount of bound PCMB-SL is proportional to the concentration of GDH in the range studied ; at the end of the reaction (2 hours) an ave-

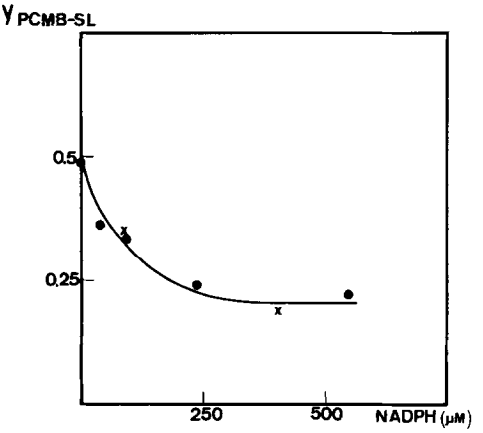

Fig. 3 : Modification of the spin-labeling yield by NADPH.
The enzyme (50 μM) was labeled in the presence of increasing NADPH concentrations. ● and x correspond to two different experiments.
$Y_{PCMB-SL}$: number of bound PCMB-SL per GDH protomer.

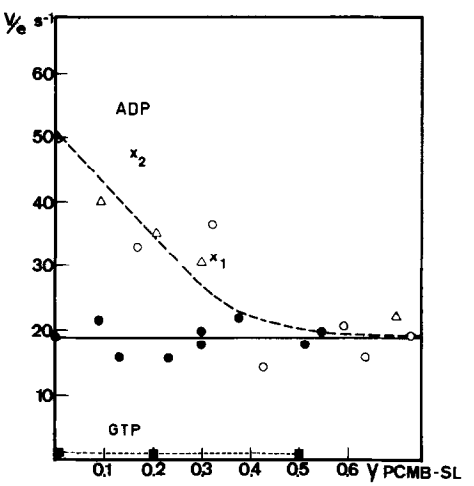

Fig. 4 : Specific activity of spin-labeled GDH, in the presence or not of effectors.
The catalytic oxidation of NADPH by 2.oxoglutarate and NH_4^+ was followed, under steady state conditions, spectrophotometrically at 340 nm.
$Y_{PCMB-SL}$: number of bound PCMB-SL per GDH protomer ; v/e is expressed in moles NADPH oxidized per mole GDH protomers and per second.
● , labeled enzyme assayed without any effector,
O , labeled enzyme assayed in the presence of 5×10^{-4} M ADP ;
■ , labeled enzyme assayed in the presence of 5×10^{-4} M GTP ;
X , enzyme assayed in the presence of 5×10^{-4} M ADP, but labeled in the presence of either 10^{-4} M NADPH (X_1) or 4×10^{-4} M NADPH (X_2).
◣, enzyme both labeled and assayed in the presence of 5×10^{-4} M ADP.

rage of only 0.60 ± 0.12 mole of PCMB-SL bound per mole protomer, in the absence of any substrate ligand (full circles).

We have observed that, if the labeling reaction is performed, under the same conditions as previously described, but in the presence of NADPH, the labeling level is significantly decreased (Fig. 2), depending on the NADPH concentration (Fig. 3), with a $K_{1/2}$ around 40 µM. The presence of NADH or ADP does not provoke such a protection effect.

The labeled enzyme, when assayed under steady state conditions, by following the catalytic oxidation of NADPH by 2-oxoglutarate and NH_4^+ in the absence of effectors GTP or ADP, shows the same specific activity whatever the amount of labeling (Fig. 4). The amount of inhibition by GTP under the same assay conditions, does not seem to be modified with the amount of labeling : 95 % inhibition is observed in the presence of 5×10^{-4} M GTP with either the native enzyme or the labeled enzyme.

The labeled enzyme is strongly modified as far as its activation by ADP is concerned ; while an activation of 250 % characterizes the native enzyme in the presence of 500 µM ADP in the assay medium, this activation decreases markedly with the amount of labeling. When an average 0.5 PCMB-SL is bound per protomer, the enzyme cannot be activated by ADP (Fig. 4).

Various arguments tend to show that the labeled site is different from the NADPH binding site (i) the labeled enzyme is fully active (ii) the affinity for NADPH is not changed as shown by fluorometric binding studies (K_d = 20 uM for the native enzyme and for the labeled enzyme at 22°C.). However, the reduced coenzyme labeled enzyme complex has at least one markedly different characteristics : we noticed that the ellipticity in the nicotinamide band of the bound reduced coenzyme is 50 % less in the complex with the labeled enzyme, suggesting a different positioning of the coenzyme in its site.

The data reported here show that PCMB-SL is bound very rigidly to probably a single type of amino acid residue which has no influence on the enzyme activity. Although this residue has not yet been identified, it is probably, because of the chemical nature of the spin label used, a cysteine residue. The stoichiometry, significantly lower than one, even in extending the time of the labeling reaction, may be due to the bulkiness and/or high hydrophobicity of the spin-label used. However it can also reflect a functional non-equivalence of the six protomers within the hexamer, a phenomenon for which indications exist in phosphate medium as reported and commented recently (16-18).

The protection effect shown by NADPH - a marked but not total decrease in the labeling yield - might be due to the structural change induced by NADPH binding which we have reported recently (19).

Although the reactive cysteine group involved in the label binding is not important for the enzyme activity, it is sensitive to structural changes that occur in the active site. We have indeed observed a further immobilization of the bound spin label spectrum in the presence of NADPH after addition, of the nucleotide GTP - which inhibits the labeled enzyme as well as the native one (Fig. 1). The same observation was reported with the ketone nitroxide probe (9). This fact should be considered together with the fact that GTP prevents the migration of substrate ligands from and towards the active site, which seems to reflect a rigidification of the protein molecule around the active site (18).

We acknowledge very simulating discussions with Drs. M. Iwatsubo and F. Labeyrie. Part of the ESR experiments were performed in Dr. Douzou's laboratory and the authors wish to thank him. This work was carried out with the financial support of D.G.R.S.T. and D.R.M.E. (Contract n° 74/1088).

REFERENCES

1. Mc Connell, H.M., and Mc Farland, G.B. (1970) Quater. Rev. Biophys. 3, 91-136.
2. Cohn, M. (1970) Quater. Rev. Biophys. 3, 61-89.
3. Frieden, C. (1971) Annual Rev. Biochem. 40, 653-696.
4. Anderson, B.M., Anderson, C.D., and Churchich, J.E. (1966) Biochemistry, 5, 2893-2900.
5. Malcolm, A.D.B., and Radda, G.K. (1970) Eur. J. Biochem. 15, 555-561.
6. Deppert, W., Jucho, F., and Sund, H. (1973) Eur. J. Biochem. 32, 76-82.
7. Wallis, R.B., and Holbrook, J.J. (1973) Biochem. J. 133, 173-182.
8. Wagner, R.E., and Hsu, C.J. (1970) Analytical Biochem. 36, 1-5.
9. Iwatsubo, M., Jallon, J.-M., and di Franco, A. (1973) in "Dynamic Aspects of Conformation Changes in Biological Macromolecules" (Sadron, C., ed.) p. 431-445, D. Reidel Publishing Company, Dordrecht.
10. Hsia, C. (1969) Ph D Thesis, University of Hawai, USA.
11. Adams, M.J., Buehner, M., Chandrascklar, K., Ford, G.C., Hackert, M.L., Everse, J., Kaplan, N.O., and Taylor, S.S. (1973) Proc. Nat. Acad. Sci. US, 70, 1968-1972.
12. Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B.-O., Tapia, O., Brändén, C.-I., and Akeson, A. (1976) J. Mol. Biol. 102, 27-59.
13. Kubo, H., Iwatsubo, M., Watari, H., and Soyama, T. (1959) J. Biochem. Tokyo, 46, 1171.
14. Olson, J.A., and Anfinsen, C.B. (1952) J. Biol. Chem. 197, 67-69.
15. Smith, T.L., Landon, M., Piszkiewics, D., Bratten, W.J., Langeley, T.J., and Melamed, M.C. (1972) Proc. Nat. Acad. Sci. US, 69, 1380-1383.
16. Dalziel, K., and Egan, R.R. (1972) Biochem. J., 126, 975-985.
17. Krause, J., Bühner, M., and Sund, H. (1974) Eur. J. Biochem. 41, 593-602
18. Jallon, J.-M. (1974) Thesis Doctorat d'Etat, University Paris-Sud, France.
19. Jallon, J.-M., Risler, Y., and Iwatsubo, M. (1975) Biochem. Biophys. Res. Com. 67, 1527-1536.